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a<sup>1</sup>  
cont

in PAPP-A2. Principally, PAPP-A2 contains an elongated zinc binding motif (HEXXHXXGXXH (SEQ ID NO:3), amino acids shown by one letter code) at position 733-743 (Figure 2). This motif and a structurally important methionine residue, are strictly conserved within the metzincins, a superfamily of zinc peptidases (Bode et al., 1993, FEBS Lett 331, 134-40; Stocker et al., 1995, Protein Sci 4, 823-40) --

Please amend the paragraph beginning at line 31 of page 50 with the following rewritten paragraph:

a<sup>2</sup>

-- Figure 1 shows the cDNA sequence (in 5'→3' orientation) corresponding to the mRNA that encodes preproPAPP-A2. Only the coding part of the sequence and the terminal stop codon (\*) is shown and is numbered 1-5376 of SEQ ID NO:1. The translated polypeptide sequence (SEQ ID NO:2) of preproPAPP-A2 is also shown. The signal peptide cleavage site was predicted using SignalP V2.0 to be after the alanine residue encoded by nt. 64-66 ((Nielsen et al., 1997, Protein Eng 10, 1-6), WWW prediction server is located at <http://genome.cbs.dtu.dk/>). The signal peptide of preproPAPP-A2 (nt. 1-66, 22 residues) is shown in bold. The nucleotide sequence of this figure represents nt. 1 to 5376 of SEQ ID NO:1. The protein sequence of this figure is illustrated as SEQ ID NO:2. --

Please amend the paragraph beginning at line 26 of page 51 with the following rewritten paragraph:

<sup>a3</sup> ~~Figure 3~~ shows the amino acid sequence of preproPAPP-A2 (SEQ ID NO:2) aligned with preproPAPP-A. The deduced amino acid sequence of preproPAPP-A2 (PA2) was aligned with the sequence of preproPAPP-A (PA) (SEQ ID NO:25) ((Haaning et al., 1996, Eur J Biochem 237, 159-63), AAC50543) using CLUSTAL W (Thompson et al., 1994, Nucleic Acids Res 22, 4673-80). Because the prepro-portion of PAPP-A did not show significant identity with the corresponding region of PAPP-A2, the alignment was manually adjusted to emphasize difference in length of pro-peptides. Arrows indicate the N-termini of the mature proteins as found earlier for PAPP-A (Kristensen et al., 1994, Biochemistry 33, 1592-8) (Glu-81), and here for PAPP-A2 (Ser-234). Putative signal peptides, strongly predicted using SignalP V2.0 (Nielsen et al., 1997, Protein Eng 10, 1-6) are shown with lower case letters. The pro-portion of PAPP-A2 contains one other candidate initiation codon corresponding to Met-168, but no signal peptide was predicted following this residue using SignalP. The sequence motifs of PAPP-A (Kristensen et al., 1994, Biochemistry 33, 1592-8) are also found in PAPP-A2: The catalytic zinc binding motif and residues of the putative Met-turn are underlined and bolded in both sequences. Lin-notch motifs (LNR1-3) and short consensus repeats (SCR-1-5) are boxed. Cysteine residus are

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a<sup>3</sup>  
cont shaded. All cysteines of mature PAPP-A are also found in PAPP-A2. In addition, the secreted form of PAPP-A2 has four cysteine residues (Cys-343, Cys-533, Cys-618, and Cys-1268) with no counterpart in PAPP-A.

Please amend the paragraph beginning at line 8 of page 53 with the following rewritten paragraph:

a<sup>4</sup> -Figure 7 shows the cDNA sequence of the PAPP-A2 mRNA coding region directly followed by the sequence of the 3'UTR. The sequence of the 3'UTR was obtained as detailed in Example 6.3. The first 5376 nucleotides of this sequence (nt. 1 - 5376) represents the coding sequence as illustrated in Figure 1 and SEQ ID NO:1 (nt. 1 - 5376). Nucleotides 5377 - 8527 of this sequence corresponds to the 3'UTR of the PAPP-A2 mRNA as illustrated in SEQ ID NO:1 (nt. 5377 - 8527).

Please amend Table 1 at line 20 of page 55 with the following rewritten Table 1:

TABLE 1. Locations of primers used for reverse transcription or PCR. The primers are listed in the order of their use.

<u>NAME</u>	<u>SOURCE<sup>a</sup></u>	<u>Nt. NUMBERS<sup>b</sup></u>	<u>SEQUENCE<sup>c</sup></u>
RT-N-mid:	AL031290	10262-10281, (4770-4789)	GCTCACACACCACAGGAATG* (SEQ ID NO:4)
PR-mid5:	AL031734	141874-141894, (1947-1967)	GGCTGATGTGCGCAAGACCTG (SEQ ID NO:5)
PR-mid3:	AL031290	10208-10229, (4716-4737)	GCATTGTATCTTCAGGAGCTTG* (SEQ ID NO:6)
PR-N5:	AL031734	102606-102628, (-)	GAAGTTGACTTCTGGTTCTGTAG (SEQ ID NO:7)

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PR-N3: - -, (2380-2400) CCCTGGGAAGCGAGTGAAGCC\* (SEQ ID NO:8)

RT-C: AL031290 62982-63006, (-) GCATTTCTTATAAGATCCTTCATGC\* (SEQ ID NO:9)

PR-C5: - -, (4180-4201) GACAGCTGTCCGTCATTGCTGC (SEQ ID NO:10)

PR-C3: AL031290 62876-62897, (-) CTTACTGCCTCTGAGGCAGTGG\* (SEQ ID NO:11)

<sup>a</sup>Accession numbers of the relevant genomic clones are given. Primers PR-N3 and PR-C5 were located in the sequence connecting hom-N and hom-C, and are therefore not represented in the databases.

<sup>b</sup>Nucleotide numbers refer to the numbering of the sequences as reported in the file with the relevant accession number. In parentheses are given the corresponding numbers of SEQ ID NO:1 (Figure 1), except for primers PR-N5, RT-C and PR-C3, not within this sequence.

<sup>c</sup>Sequences are actual primer sequences (orientation 5'-to-3'). Sequences marked with an asterisk are complementary to the database sequences or the sequence given in Figure 1--

Please amend the paragraph beginning at line 26 of page 57 with the following rewritten paragraph:

A cluster of EST sequences matching the genomic sequence of AL031290 were identified around nt 64000-66000 of AL031290, starting approximately 1.2 kb from the end of the PAPP-A2 encoding sequence. The existence of mRNA connecting the coding region of PAPP-A2 and this cluster was verified in a PCR using primers from AL031290 (5'-GGAAAGAGCAGAGTTCACCCAT-3' (SEQ ID NO:12), nt. 64900-64879 of AL031290) and the PAPP-A2 encoding sequence (5'-CCGTCTTAGTCCACTGCATCC-3' (SEQ ID NO:13), nt. 20499-20519 of AL031290, nt 5171-5191 of AF311940), and oligo-dT primed placental cDNA as a template (Overgaard et al., 1999, Biol Reprod 61, 1083-9). As expected, the size of the resulting product was 2.2 kb,

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further demonstrating the existence of a PAPP-A2 mRNA with a 3'UTR of about 3 kb. The distribution among tissues is shown in Table 2. f

Please amend the paragraph beginning at line 30 of page 59 with the following rewritten paragraph:

Construction of pPA2-KO: The construct pPA2-KO is a variant of the pPA2 expression construct in which residue Glu-734 of the active site of PAPP-A2 was substituted with a Gln residue. Thus, the mutant is E734Q. The pPA2-KO construct was made by site directed mutagenesis using the method of overlap extension PCR (Ho et al., 1989, Gene 77, 51-9) with pPA2 as the template. In brief, outer primers were 5'-CGCTCAGGGAAGGACAAGGG-3' (5' end primer, nt. 976-995 of SEQ ID NO:1) and 5'-CTAGAAGGCACAGTCGAGGC-3' (SEQ ID NO:14) (3' end primer, nt. 1040-1021, sequence of vector pCDNA3.1+). Overlapping internal primers were 5'-TGTCCTCACTTGATGGATCATGGTGTCTCGGTGTGG-3' (SEQ ID NO:15) (nt. 2210-2178 of SEQ ID NO:1, nt. 2200 not C, but G resulting in E734Q) and 5'-CCATCAAGTGGGACATGTTCTGGGAC-3' (SEQ ID NO:16) (nt. 2196-2221 of SEQ ID NO:1, nt. 2200 not G, but C resulting in E734Q). The resulting mutated fragment was digested with *XbaI* and *XhoI* and swapped into pPA2 to generate pPA2-KO. All PCRs were carried out with *Pfu* DNA polymerase (Stratagene), and all constructs were verified by sequence analysis. f

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Please amend the paragraph beginning at line 11 of page 60 with the following rewritten paragraph:

*Q8* Construction of pPA2-mH: Two primers (5'-GAGGGCCTGTGGACCCAGGAG-3', nt. 4906-4926 of SEQ ID NO:1, and 5'-GACGTAAAGCTTCTGATTTTCTTCTGCCTTGG-3' (SEQ ID NO:17), nt. 5373-5354 of SEQ ID NO:1, preceded by a *HindIII* site, AAGCTT, and nt. GACGTA to facilitate cleavage of the PCR product) were used in a PCR with pPA2 as the template to generate a nucleotide fragment encoding the C-terminal 156 residues of PAPP-A2 with the stop codon replaced by a *HindIII* site for in-frame ligation to expression vector. In brief, the PCR product was digested with *EcoRI* and *HindIII* and cloned into the *EcoRI/HindIII* sites of the vector pcDNA3.1/Myc-His(-)A to generate pPA2C-mH. The *NotI-XbaI* fragment (encoding the N-terminal portion of PAPP-A2), and the *XbaI-EcoRI* fragment (encoding the remaining central portion of PAPP-A2) were excised from pPA2 and ligated in one reaction into the *NotI/EcoRI* sites of pPA2C-mH. The resulting construct, pPA2-mH, encoded PAPP-A2 followed by residues KLGP (SEQ ID NO:18), the myc epitope (EQKLISEEDL (SEQ ID NO:19)), residues NSAVD (SEQ ID NO:20), and six H-residues (amino acids are given as one letter code). A stop codon follows immediately after the six histidine residues.

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Please amend the paragraph beginning at line 28 of page 61 with the following rewritten paragraph:

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 -C-terminally tagged PAPP-A2 purified from medium of cells transfected with construct pPA2-KO-mH (see examples 6.4 and 6.5) was reduced and run on a 10-20% SDS gel, and further blotted onto PVDF membrane (ProBlott, Applied Biosystems). Bands of 4 lanes were excised and subjected to N-terminal sequence analysis on an Applied Biosystems 477A sequencer equipped with an on-line HPLC (Sottrup-Jensen, 1995, Anal Biochem 225, 187-8). The N-terminal sequence observed at a level of approximately 20 pmol was: Ser-Pro-Pro-Glu-Glu-Ser-Asn (SPPEESN) (residues 234-240 of SEQ ID NO:2), resulting from cleavage before Ser-234 of the PAPP-A2 polypeptide after R(230)VKK (residues 230-233 of SEQ ID NO:2).-

Please amend the paragraph beginning at line 21 of page 62 with the following rewritten paragraph:

A<sup>10</sup>  
 -For further analysis, recombinant IGFBP-5 was produced in mammalian cells. In brief, human placental oligo-dT primed cDNA (Overgaard et al., 1999, Biol Reprod 61, 1083-9) was used as a template to amplify cDNA encoding human IGFBP-5 (Accession number M65062). Specific primers containing an *XhoI* site (5'-TCCGCTCGAGATGGTGTGCTCACCGCGGT-3' (SEQ ID NO:21)) and a *HindIII* site (5'-CGATAAGCTTCTCAACGTTGCTGCTGTCG-3' (SEQ ID NO:22)) were used,

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and the resulting PCR product was digested and cloned into the *XhoI/HindIII* sites of pcDNA3.1/*Myc-His(-)A* (Invitrogen). The construct encoded the full-length proIGFBP-5, immediately followed by residues KLGP, the *myc* epitope (EQKLISEEDL (SEQ ID NO:19)), residues NSAVD (SEQ ID NO:20), and six H-residues (amino acids are given as one letter code). The construct was verified by sequence analysis. Plasmid DNA for transfection was prepared by QIAprep Spin Kit (Qiagen). Cell culture and expression of recombinant IGFBP-5 was performed as described above in Example 6.4.-

Please amend the paragraph beginning at line 22 of page 63 with the following rewritten paragraph:

a<sup>11</sup>  
- For cleavage site determination, purified rIGFBP-5 (Fig. 6, lane 7) was digested with purified PAPP-A2 and analyzed by SDS-PAGE (Fig. 6, lane 8). Edman degradation of blotted material showed that both distinct, visible degradation products (fig. 6, lane 8) contained the N-terminal sequence K(144)FVGGA (SEQ ID NO:23) (IGFBP-5 is numbered with the N-terminal Leu of the mature protein as residue 1). The two bands both represent intact C-terminal cleavage fragments, because they also contain the C-terminal c-myc tag (Fig. 6, lane 9); they are likely to be differently glycosylated, in accordance with the heterogeneity of purified rIGFBP-5 (Figure 6, lane 7). Both bands contained a second sequence at lower



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level (45%), L(1)GXFVH (SEQ ID NO:24), corresponding to the N-terminal sequence of IGFBP-5. The absence of Ser, expected in the third cycle, was taken as evidence for carbohydrate substitution of Ser-3. O-linked glycan on the N-terminal cleavage fragment is likely to cause it to smear around the two distinct, C-terminal fragments. Sequence analysis on the reaction mixture (> 100 pmol) without SDS-PAGE separation showed only the same two IGFBP-5 sequences in equimolar amounts. Thus, PAPP-A2 cleaves IGFBP-5 at one site, between Ser-143 and Lys-144. -

IN THE SEQUENCE LISTING

Please substitute the attached Sequence Listing, numbered as pages 1-32 for the Sequence Listing previously submitted.